

クロチョウガイのヘモリンパ液における二酸化炭素分圧と炭酸水素イオン濃度の算出

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Estimation of CO₂ partial pressure and bicarbonate concentration in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera*

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Abstract : We examined hemolymph pH, total CO₂ content (Tco₂, mM/l), CO₂ partial pressure (Pco₂, mmHg) and bicarbonate concentration ([HCO₃⁻], mM/l) in order to evaluate the acid-base balance of the black-lip pearl oyster *Pinctada margaritifera* under normoxic conditions. Hemolymph was collected anaerobically through a cannula after catheterization of the adductor muscle. The mean values of the hemolymph pH and Tco₂ were 7.563 and 2.04 mM/l, respectively. Using the CO₂ solubility coefficient and apparent dissociation constant of carbonic acid determined in this study, Pco₂ and [HCO₃⁻] were calculated as 1.50 mmHg and 1.98 mM/l, respectively. These values were in same range as those of the hemolymph of the akoya pearl oyster *P. fucata*.

Key words : *Pinctada margaritifera*, cannulation, hemolymph, acid-base balance, Pco₂, dissociation constant of carbonic acid

Introduction

The black-lip pearl oyster *Pinctada margaritifera* is a filibranchial bivalve classified in the Pteriidae.¹⁾ *P. margaritifera* is distributed in the Indo-West Pacific region near the equator, including in the Red Sea, Arabian Sea, Persian Gulf, and around India, Sri Lanka, New Guinea, Hawaii and Madagascar.²⁾ In Japan, *P. margaritifera* is present southwards from the Kii Peninsula,¹⁾ where the annual seawater temperatures in the coastal zone of Wakayama, Kochi and Okinawa prefectures are 15–31°C.³⁾

The black-lip pearl oyster has nacreous aragonite in the inner layer of its shell valves, and it is used for black pearl production worldwide. The process of pearl production is similar to the growth of the shell valves and is directly related to metabolism. The metabolism of the black-lip pearl oyster has been studied in terms of regulation of gill ventilation volume and oxygen uptake under normoxic, hypoxic and anathermal conditions.⁴⁻⁷⁾ The anatomical structures of the ctenidium, circulatory system, digestive diverticula and labial palp have been

studied.^{8,9)} However, there are few reports on the respiratory mechanism from the viewpoint of CO₂ dynamic phase and acid-base balance. Research into the acid-base status could contribute to efficient CO₂ utilization, which is related to calcification for pearl formation and growth of the shell valve. The estimated CO₂ partial pressure of the hemolymph was 0.9 mmHg in sea mussel *Mytilus edulis*,¹⁰⁾ 2.3 mmHg in Asian freshwater clam *Corbicula fluminea*,¹¹⁾ and 1.7–2.3 mmHg in akoya pearl oyster *P. fucata*.^{12,13)} Because the CO₂ partial pressures in bivalves are very low, it was supposed that the CO₂ partial pressure in the black-lip pearl oyster would also be similarly very low; however, the direct measurement of CO₂ partial pressure is difficult. The estimation of CO₂ partial pressure by application of the Henderson–Hasselbalch equation is practiced in studies of the acid-base balance owing to the relative ease and accuracy of the estimates.¹⁴⁾ In the equation, the characteristic values of the CO₂ solubility coefficient (α_{CO_2}) and apparent dissociation constant of carbonic acid (pKapp) in the hemolymph are required for the experimental animal. Therefore, we examined

black-lip pearl oyster hemolymph pH, total CO₂ content, CO₂ partial pressure and bicarbonate concentration using the hemolymph α CO₂ and pK_{app}, which were determined in this study. By means of catheterization of the adductor muscle, hemolymph was collected anaerobically from black-lip pearl oysters underwater.

Materials and Methods

Experimental animals and conditions

The experiments used 78 black-lip pearl oysters (shell length: 90.4 ± 1.8 mm (Mean \pm SE), shell height: 108.1 ± 1.9 mm, and total wet weight: 188.2 ± 10.5 g). The animals were obtained from the coast of Kochi prefecture, Japan. After cleaning the shell valves, they were reared for one month at 26°C in aerated seawater with added cultivated phytoplankton.^{13,15} Twenty-four hours before collecting hemolymph, the black-lip pearl oysters were transferred to seawater that was particle-free (>0.45 μ m). All experiments were conducted in seawater with a salinity of 33 psu, water temperature 26°C, O₂ saturation 98%, pH 8.10, and total CO₂ content 1.8 mM/l.

Surgical procedures and hemolymph collection

Hemolymph was collected from the adductor muscle using a cannula (polyethylene tubing, 0.96 mm outer diameter, 0.58 mm inner diameter, PE-50, Clay Adams). A small hole (2 mm diameter) was made on adjacent shell valves, which was at the center of the posterior margin. The cannula with a stylet was inserted through the hole into the adductor muscle, which is the part of the large anterior muscle, and was advanced 0.7–1.0 cm toward the center of the adductor muscle. The stylet was removed, and the end of the cannula was closed. The cannula was gently fixed to the left shell valve using denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent effects from movement of the shell valves. This surgical operation was completed within 5 minutes. The cannulated oyster was transferred to a darkened acrylic respiratory chamber, and was allowed to recover for 2 hr at 26°C under normoxic conditions. A hemolymph sample was then drawn through the cannula

using a gas-tight micro syringe (Model 1750, Hamilton Co.). The volume of hemolymph collected was 0.5 ml.

Hemolymph analysis

The hemolymph pH and total CO₂ content (Tco₂, mM/l) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200, Cameron Instruments) using pH glass and reference electrodes (E301, E351, Cameron Instruments) at 26.0°C. Tco₂ was measured using a total CO₂ analyzer (Capnicon 5, Cameron Instruments). The hemolymph CO₂ partial pressure (Pco₂, mmHg) and bicarbonate concentration ([HCO₃⁻], mM/l) were calculated by rearranging the Henderson-Hasselbalch equation.^{14,16} In the equation, the CO₂ solubility coefficient (α CO₂, μ M/l/torr) and apparent dissociation constant of carbonic acid (pK_{app}) of black-lip pearl oysters were required. The determinations of α CO₂ and pK_{app} were performed *in vitro*.

α CO₂ was determined using hemolymph, which was adjusted to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Industries, Ltd.). The acidified sample was transferred to a tonometer flask, and equilibrated with humidified standard CO₂ gas (CO₂, 15.0%; O₂, 20.9%; N₂ Balance) using an equilibrator (DEQ-1, Cameron Instruments) at 26.0°C, and subsequently the total CO₂ content of each equilibrated sample was measured with a total CO₂ analyzer. The CO₂ partial pressure of the equilibrated sample was calculated from known CO₂ concentration standard gas (15.0%), prevailing barometric pressure and water vapor pressure at 26.0°C. The CO₂ solubility coefficient was calculated using the equation:

$$\alpha\text{CO}_2 = \text{Total CO}_2 \text{ content} \cdot \text{CO}_2 \text{ Partial pressure}^{-1}$$

For determination of the apparent dissociation constant of carbonic acid, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO₂ gases (CO₂, 0.2, 0.5, 1.0, 2.0 and 5.0%; O₂, 20.9%; N₂ Balance) using an equilibrator at 26.0°C. After equilibration, the pH and total CO₂ content of the sample were measured with a blood gas meter and a total CO₂ analyzer. Using the sample pH, total CO₂ content and α CO₂ calculated by the above equation, the pK_{app} was

determined by rearrangement of Henderson-Hasselbalch equation^{14,16} as follows:

$$pK_{app} = pH - \log \left[\frac{\text{Total CO}_2 \text{ concentration} - \alpha\text{CO}_2 \cdot \text{CO}_2 \text{ Partial pressure}}{\alpha\text{CO}_2 \cdot \text{CO}_2 \text{ Partial pressure}} \right]^{-1}$$

where CO₂ partial pressure is calculated from known CO₂ concentration standard gases.

The αCO_2 and pK_{app} obtained in this study were used for calculation of hemolymph Pco₂ from measured pH and Tco₂:

$$P_{\text{CO}_2} = T_{\text{CO}_2} \cdot [\alpha\text{CO}_2 \cdot (1 + 10^{(pH - pK_{app})})]^{-1}$$

[HCO₃⁻] was calculated from Tco₂, αCO_2 and Pco₂ using the equation:

$$[\text{HCO}_3^-] = T_{\text{CO}_2} - \alpha\text{CO}_2 \cdot P_{\text{CO}_2}$$

Statistical analysis

All data are expressed as means \pm standard error. Analysis of variance was used to test for changes in hemolymph properties using the standard CO₂ gases. Unpaired t-test was used for the comparison of mean values of hemolymph parameters. Statistically significant differences were set at $P < 0.01$.

Results

Hemolymph samples were collected from the adductor muscles of black-lip pearl oysters through cannulae. The collection volume was 0.4–0.5 ml. The hemolymph pH and Tco₂ under normoxic conditions were 7.563 ± 0.0142 and 2.04 ± 0.058 mM/l, respectively. The hemolymph αCO_2 was 36.27 ± 0.740 $\mu\text{M/l/mmHg}$. The hemolymph pK_{app} at known CO₂ partial pressures (standard gases) and the corresponding measured pH and Tco₂ values are shown in Table 1. The calculated pK_{app} from all hemolymph samples was 5.99878 ± 0.01756 . Pco₂ and [HCO₃⁻] were calculated by substitution of the mean value of hemolymph αCO_2 and pK_{app} in the rearranged Henderson-Hasselbalch equation as follows:

$$P_{\text{CO}_2} = T_{\text{CO}_2} \cdot [0.03627 \cdot (1 + 10^{(pH - 5.99878)})]^{-1}$$

$$[\text{HCO}_3^-] = T_{\text{CO}_2} - 0.03627 \cdot P_{\text{CO}_2}$$

where the units of the parameters at the equations are mmHg in Pco₂, mM/l in Tco₂ and mM/l in [HCO₃⁻].

Hemolymph Pco₂ and [HCO₃⁻] at 26°C under normoxic condition were 1.50 ± 0.065 mmHg and 1.98 ± 0.056 mM/l, respectively (Table 2). In *in vitro* experiments (Table 1), the pH decreased significantly with the increase in Pco₂ ($P < 0.01$). At the same time, the interaction between pK_{app} and pH was analyzed (Fig. 1), and the correction equation of pK_{app} was obtained as follows:

$$pK_{app} = 174.2725 - 73.0837 \cdot pH + 10.5906 \cdot pH^2 - 0.5118 \cdot pH^3$$

For the comparison, using the hemolymph pH and Tco₂ measured immediately after collection, Pco₂ and [HCO₃⁻] were estimated by the correction equation and are shown in Table 3. The hemolymph Pco₂ calculated from the mean value of pK_{app} was higher than that by the correction equation ($P < 0.01$, Table 3). There was no difference in hemolymph [HCO₃⁻] calculated by the two methods.

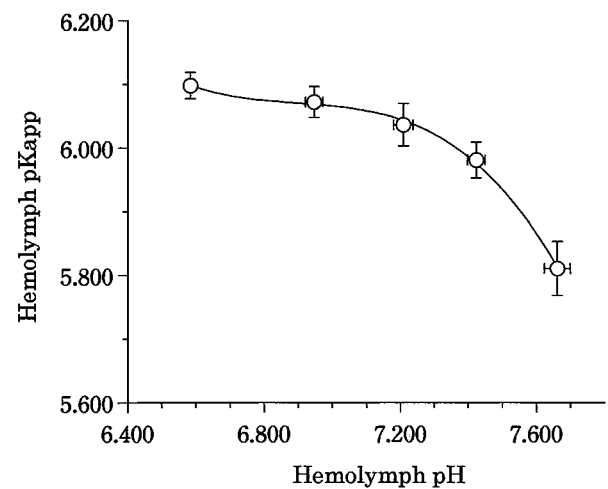


Fig. 1. Relationship between pH and apparent dissociation constant of carbonic acid (pK_{app}) in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera* at 26°C. Values are means \pm SE (N=15). Solid line fitted to the data and the equation: $pK_{app} = 174.2725 - 73.0837 \cdot pH + 10.5906 \cdot pH^2 - 0.5118 \cdot pH^3$ ($r=0.99$)

Table 1. Mean values of measured pH, total CO₂ content (Tco₂) and calculated apparent dissociation constant of carbonic acid (pKapp) of the hemolymph in adductor muscle of the black-lip pearl oyster (*Pinctada margaritifera*) with known Pco₂ standard gases

Standard gas		Hemolymph			
CO ₂ (%)	Pco ₂ (mmHg)	pH	Tco ₂ (mM/l)	pKapp	N
0.2	1.49	7.661	3.884	5.8107	15
0.5	3.72	7.422	3.874	5.9810	15
1.0	7.44	7.207	4.297	6.0358	15
2.0	14.88	6.946	4.602	6.0718	15
5.0	37.20	6.582	5.477	6.0982	15

Barometric pressure 765 mmHg, Water temperature 26.0°C

Table 2. Hemolymph pH, total CO₂ content (Tco₂), CO₂ partial pressure (Pco₂) and bicarbonate concentration ([HCO₃⁻]) of the black-lip pearl oyster (*Pinctada margaritifera*) at 26°C under normoxic condition

	Mean	SE	N	
pH	7.563	0.0142	18	
Tco ₂	mM/l	2.04	0.058	18
Pco ₂	mmHg	1.50	0.065	18
[HCO ₃ ⁻]	mM/l	1.98	0.056	18

αco₂, 36.27 μM/l/mmHg; pKapp, 5.99878

Table 3. The comparison of the hemolymph Pco₂ and [HCO₃⁻] calculated by the mean value of pKapp and by the correction equation

	Pco ₂ (mmHg)	[HCO ₃ ⁻] (mM/l)	N
the mean value of pKapp	1.50	1.98	18
the correction equation of the pKapp	1.20 *	1.99	18

* : statistically significant difference (unpaired *t*-test, *P*<0.01)

Discussion

We collected hemolymph from the adductor muscle and examined hemolymph pH, total CO₂ content, CO₂ partial pressure and bicarbonate concentration in order to evaluate the acid-base balance of black-lip pearl oysters. The hemolymph collected anaerobically through a cannula from black-lip pearl oysters underwater by pretreatment of the adductor muscle catheterization. The mean values of pH and Tco₂ measured immediately after hemolymph collection were 7.563 and 2.04 mM/l, respectively. The mean values of hemolymph pH were 7.65 in sea mussel *M. edulis* at 12°C,¹⁰⁾ 7.36 in Pacific oyster *Crassostrea gigas* at 15°C,¹⁷⁾ 7.55 in *M. galloprovincialis* at 18°C,¹⁸⁾ and 7.284–7.375 in the akoya pearl oyster *P. fucata* at 28°C.¹³⁾ Although there are few descriptions of the hemolymph total CO₂ content in marine bivalves, Handa and Yamamoto (2011, 2012) reported that the mean value in the akoya pearl oyster *P. fucata* was 1.90–2.10 mM/l.^{12,13)} The hemolymph pH and Tco₂ in the black-lip pearl oyster were almost the same as those in other marine bivalves.

Cameron (1986) reported the CO₂ solubility of seawater as a function of temperature, and the solubility coefficient was 35.49–38.12 μM/l/mmHg at 26–28°C.¹⁹⁾ The hemolymph αCO₂ in the black-lip pearl oyster (36.27 μM/l/mmHg) was in the range of the coefficient of seawater. The mean value of the hemolymph pK_{app} in this study was 5.99878, whereas that in the adductor muscle of akoya pearl oyster hemolymph at 28°C was 5.8191,¹³⁾ and that in hemolymph of sea mussel *M. edulis* at 12°C was 6.114.^{10,20)} The apparent dissociation constant of carbonic acid is equal to the pH at which it shows the most effective as a buffer.²¹⁾ The most effective buffer of the black-lip pearl oyster hemolymph seemed to be lower than that in sea mussel *M. edulis*, and was consistent with that in the akoya pearl oyster *P. fucata* which is classified into the same genus as the black-lip pearl oyster.

Using the αCO₂ and pK_{app} in this study, Pco₂ and [HCO₃⁻] were calculated. The mean values of hemolymph

Pco₂ and [HCO₃⁻] were 1.50 mmHg and 1.98 mM/l, respectively (Table 2). In some marine bivalves, mean values of hemolymph Pco₂ and [HCO₃⁻] were 0.9 mmHg and 1.8 mM/l in sea mussel *M. edulis* at 12 °C¹⁰⁾, 0.15 kPa (2.0 mmHg) and 1.37 mM/l in Pacific oyster *C. gigas* at 15°C¹⁷⁾, 1.15 mmHg and 1.62 mM/l in *M. galloprovincialis* at 18°C¹⁸⁾, and 2.08–2.33 mmHg and 1.83–2.04 mM/l in the akoya pearl oyster *P. fucata* at 28°C.¹³⁾ The hemolymph acid-base status in the black-lip pearl oyster was similar to those in other marine bivalves. In teleosts, the blood Pco₂ in the carp *Cyprinus carpio* was 3.8–4.2 mmHg,^{22,23)} which is 10% that of humans (Pco₂ = 40 mmHg). The carp blood Pco₂ suggested that CO₂ is excreted rapidly from the blood, which enables common carp to live in ambient water where pH and dissolved gases often vary.^{22,23)} Therefore, the black-lip pearl oyster should excrete or utilize CO₂ rapidly from the hemolymph, which then regulates the acid-base balance and calcification for shell valves and pearl formation.

On the other hand, the pH decreased significantly with the increase in Pco₂ (Table 1). The relationship of hemolymph pH and pK_{app} was shown, and Pco₂ and [HCO₃⁻] were estimated on the basis of the correction equation. The hemolymph Pco₂ estimated by the correction equation was lower than that from the mean value of pK_{app}, nevertheless there was no difference in [HCO₃⁻] (Table 3). αCO₂ and pK_{app} vary with ionic strength and temperature,¹⁴⁾ and estimation of the Pco₂ could be affected by temperature and salinity. Therefore, it is necessary to examine these parameters at various temperatures and salinities in order to increase the accuracy of the calculation of Pco₂ and to formulate the correction equation for pH, salinity and water temperature.

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クロチョウガイのヘモリンパ液における二酸化炭素分圧と炭酸水素イオン濃度の算出

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要 旨

クロチョウガイの酸塩基平衡を明らかにするため、クロチョウガイ閉殻筋からポリエチレン細管を通してヘモリンパ液を嫌氣的に採取し、pHと全炭酸含量を測定した。さらに二酸化炭素分圧と炭酸水素イオン濃度を算出するため、クロチョウガイヘモリンパ液の二酸化炭素溶解度と炭酸の解離定数を分析した。その結果、水温26度におけるクロチョウガイのヘモリンパ液pHは7.563(平均値)、全炭酸含量は2.04 mM/lと測定された。本研究で分析した二酸化炭素溶解度と炭酸解離定数を用いて二酸化炭素分圧と炭酸水素イオン濃度を算出すると、それぞれ1.50 mmHg、1.98 mM/lを示した。これらの値は、アコヤガイのヘモリンパ液での値に近似した。